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PRINCIPAL INVESTIGATOR: Pui Kai Li, Ph.D.

CONTRACTING ORGANIZATION: Ohio State University
Columbus, OH 43210-1239

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Pui Kai Li, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Ohio State University
Columbus, OH 43210-1239

E-Mail: li.27@osu.edu

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13. ABSTRACT (Maximum 200 Words)

Purpose: To design dual acting inhibitors that can block the enzyme estrone sulfatase and act as antiestrogens.
Scope: The design and synthesis of 30 dual inhibitors are proposed. The inhibitors contain 4 different structural core. The synthesized inhibitors will be tested on their ability to inhibit the enzyme estrone sulfatase and also their abilities to inhibit the growth of breast cancer cells stimulated by estrone sulfate. In addition, selected inhibitors will be tested in vivo using NMU-induced mammary tumors in rats.
Major findings: More than 83 % (25 out of 30) of the proposed inhibitors have been synthesized. The inhibitors have been tested for their ability to inhibit estrone sulfatase activity of rat liver microsomes at 20 μM concentrations and in the presence of 20 μM of substrate estrone sulfate. All the inhibitors tested so far are more potent than our lead compound Tamoxifen sulfamate. Raloxifene sulfamate (inhibitor **30**) is still the most potent compound among the 25 inhibitors we have synthesized. It inhibits more than 95% of the sulfatase activity at 20 μM concentration. It is by far the most potent dual inhibitor we have ever obtained. We have selected inhibitor **30** as one of the compounds for in vivo study using NMU-induced mammary tumors in rats. We have synthesized 4 grams of the compound needed for the study.

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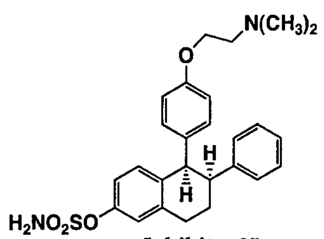
Introduction

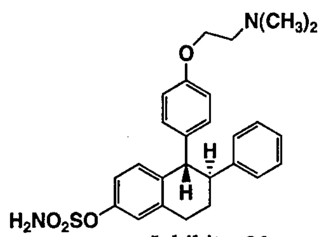
Breast cancer is the most common malignancy in the United States. It is estimated that approximately 30 - 40 % of all breast cancers are estrogen-dependent. Currently, the most common treatments use either antiestrogen or aromatase inhibitors. They are effective in 35-40 % of advanced postmenopausal breast cancer patients. In estrogen-dependent breast cancer patients, the estrogen levels in breast cancer cells are 5-10 times higher than in plasma. One of the possibilities to explain this observation is *in situ* production of estrogens from precursor substrates in the breast cancer cells. One of the pathways for the *in situ* production of estrogen is the conversion of androgens to estrogens by the enzyme aromatase (aromatase pathway). Another pathway for the *in situ* formation of estrogen is through the conversion of estrone sulfate to estrone by the enzyme estrone sulfatase (estrone sulfatase pathway). It has been pointed out that the estrone sulfatase pathway is significant and produce 10 times more estrogen than through the aromatase pathway in breast cancer cells. In addition, estrone sulfatase is also responsible for the conversion of dehydroepiandrosterone sulfate to androst-5-ene-3β,17β-diol, another estrogenic steroid in the body. Thus, potent estrone sulfatase inhibitors are potential agents for the treatment of estrogen-dependent breast cancer. Preliminary studies demonstrated that estrone sulfatase inhibitor can block the growth of NMU-induced tumor in rat stimulated by estrone sulfate. Thus the current approach is to design dual acting inhibitors that can not only block the estrone sulfatase pathway, but also act as antiestrogens. The proposed dual acting inhibitors will have advantage over the current drug treatments. The inhibitors will not only block the formation of estrogen, but also block the stimulatory effect of estrogen on cancer cells. This proposal will design and synthesize of dual acting inhibitors with sulfatase inhibitory and anti-estrogenic activity. The synthesized inhibitors will be tested using enzyme inhibition and cell culture assays. Finally, In vivo studies of dual acting inhibitors using NMU-induced mammary tumor in rats will be performed.

Body

As stated in the introduction, this proposal deals with the design, synthesis and biological testings of dual inhibitors with sulfatase inhibitory and anti-estrogenic activities. A total of 30 inhibitors are proposed. In the first year we have synthesized 16 inhibitors (inhibitors **1-15** and **30** stated in the proposal). In the second year, we completed the synthesis of inhibitors **16-24** proposed in the grant. The remaining compounds (inhibitors **25-29**, structures shown below) are completed this year.



Inhibitor 25



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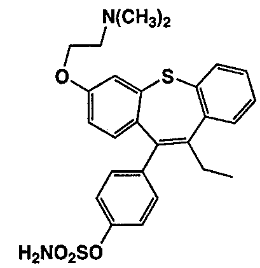

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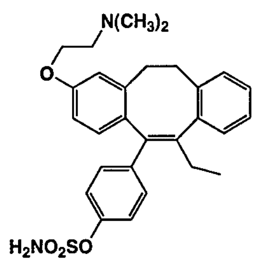

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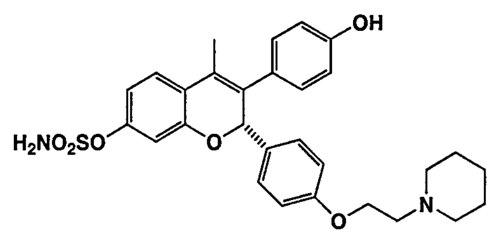


Inhibitor 29

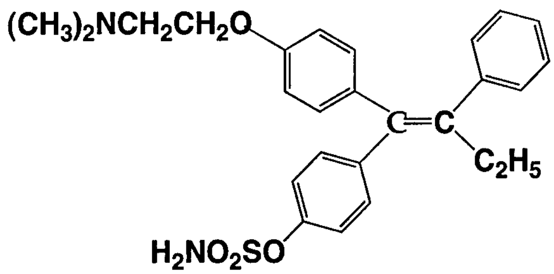
The syntheses of inhibitors **25-29** carried out according to the literature except the sulfamoylation step. We did not come across any difficulty.

Enzyme Inhibition studies of inhibitors

Inhibitors **25 - 29** were tested for their abilities to inhibit estrone sulfatase activity of rat liver microsomes at 20 μM concentrations and in the presence of 20 μM substrate estrone sulfate. At 20 μM inhibitor concentration, the % inhibiton of sulfatase activity activity range from 9 – 42 %. Raloxifene sulfamate (inhibitor **30**) is still the most potent inhibitor among all the inhibitors we synthesized (over 95% inhibition) at the same inhibitor concentration.

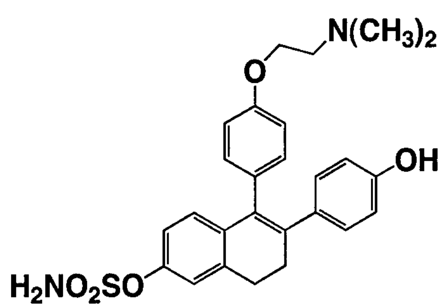
When compared all the inhibitors we synthesized, we choose Tamoxifen sulfamate, compound **7** and compound **30** and determine their IC_{50} values on sulfatase inhibition (shown below).

IC_{50} of steroid sulfatase



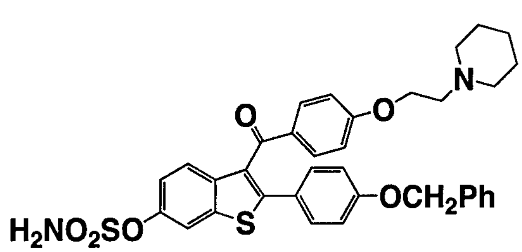
35.9 μM

(E)-Hydroxytamoxifen sulfamate



4.4 μM

Inhibitor 7



0.06 μM

Inhibitor 30

We have reported the first inhibitor (E-hydroxytamoxifen sulfamate) with dual activity, inhibiting both estrone sulfatase and also act as antiestrogen. However, E-hydroxytamoxifen sulfamate has weak sulfatase inhibitory activity. In addition, the active estrogen, Z-4-hydroxytamoxifen can be isomerized to the inactive E-hydroxytamoxifen. One alternative to prevent the isomerization is incorporating the ethyl group in the hydroxytamoxifen into a ring such as nafoxidine and diphenylbenzocycloheptene. When compared the sulfatase inhibitory activity of E-Hydroxytamoxifen with one of the analogs of nafoxidine sulfamate (compound 7), the sulfatase inhibitory activity of compound 7 is approximately 8 fold more potent. Most amazingly, replacing the dihydronaphthalene nucleus in compound 7 with a benzothiophene nucleus to form compound **30**, the sulfatase inhibitory activity of compound 30 increases another 73 fold and has an IC_{50} value of 60 nM. Compound **30** is the most potent sulfatase inhibitor among all 30 compounds.

We have requested for a one year no cost extension to complete the in vivo antitumor study of compound **30** on NMU induced mammary tumor in rat.

Key Research Accomplishment

All the proposed inhibitors (compounds 1 – 30) have been synthesized. All the inhibitors tested so far are more potent than our lead compound E-hydroxytamoxifen sulfamate. Raloxifene sulfamate (inhibitor **30**) exhibits an extremely potent sulfatase inhibitory activity. It inhibits more than 95% of the sulfatase activity at 20 μM concentration and exhibit an IC_{50} value of 60 nM. It is by far the most potent dual inhibitor we have ever obtained and also the most potent dual inhibitor ever reported in the literature. We have synthesized 4 grams of inhibitor **30** for in vivo antitumor study.

Reportable Outcomes

1. A manuscript has been prepared on the synthesis and sulfatase inhibitory activities of dual inhibitors with nafoxidine nucleus (please refer to the appendix).

2. Presentation of poster "DESIGN AND SYNTHESES OF DUAL ACTING INHIBITORS FOR BREAST CANCER" at the Era of Hope, Department of Defense Breast Cancer Research Program Meeting, September, 25-28, 2002, Orlando Florida. (abstract attached)

3. Oral presentation at the Gordon Conference "Hormonal Carcinogenesis" July 6-11, 2003, Meriden, NH. Seminar title "Estrogen sulfatase and inhibitors in breast cancer".

Personnel receiving pay from the project

Pui-Kai Li

Kyle Selcer

JianDong Shi

Zili Xiao

Jennifer Sarap

Conclusions:

All 30 proposed inhibitors have been synthesized and tested for their ability to inhibit estrone sulfatase activity of rat liver microsomes at 20 μM concentrations and in the presence of 20 μM substrate estrone sulfate. The inhibitors belong to the nafoxidine, benzocyclohepterne and raloxifene structural classes. All the inhibitors showed significant inhibition of estrone sulfatase and are more potent than our lead compound Tamoxifen sulfamate. Raloxifene sulfamate (inhibitor **30**) exhibits an extremely potent sulfatase inhibitory activity and has been chosen to be one of the compounds for in vivo anti-tumor study. We have synthesized 4 grams of the compound.

APPENDIX COVER SHEET

DESIGN AND SYNTHESES OF DUAL ACTING INHIBITORS FOR BREAST CANCER

Pui-Kai Li, Jiandong Shi, Guo-Hua Chu, Rucha Sane, Jennifer Sarap, Heidi Kabler, Kyle W. Selcer

Division of Med. Chem and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus OH 43210, Department of Medicinal Chemistry, Mylan School of Pharmacy and Department of Biology, School of Natl. & Environ. Sci., Duquesne University, Pittsburgh PA 15282.

E-mail li.27@osu.edu

ABSTRACT: Estrogen levels in breast tumors of post-menopausal women are at least ten times higher than estrogen levels in plasma. The high levels of estrogen in these tumors are presumably due to in situ formation of estrogen, possibly through conversion of estrone sulfate to estrone by the enzyme estrone sulfatase. Therefore, inhibitors of estrone sulfatase are potential agents for the treatment of estrogen-dependent breast cancers. Among all the estrone sulfatase inhibitors, estrone-3-O-sulfamate (EMATE) and its analogs are the most potent. EMATE is classified as an active-site directed irreversible inhibitor. Recently, non-steroidal estrone sulfatase inhibitors were developed based on the fact that EMATE was found to be estrogenic. Non-steroidal sulfatase inhibitors such as coumarin sulfamate and (p-O-sulfamoyl)-N-tetradecanoyl tyramine were reported to inactivate estrone sulfatase in an active-site directed manner. It can be concluded that the common functionality for sulfatase inactivation is a phenylsulfamoyl group.

We synthesized (E)-4-hydroxytamoxifen sulfamate as dual inhibitor (inhibitor with sulfatase inhibitor activity and antiestrogenic activity). (E)-Hydroxytamoxifen sulfamate competitively inhibited estrone sulfatase and exhibited apparent Ki of 35.9 ± 4.4 micromolar. It has higher affinity than the substrate estrone sulfate since the Km of the substrate is 90.2 ± 8.0 micromolar. Eight sulfamate analogs with nafoxidine nucleus were synthesized. In addition, four sulfamate analogs with raloxifene nucleus were also synthesized as dual acting agents.

The dual inhibitors were tested for their abilities to inhibit estrone sulfatase activity of rat liver microsomes. The most potent nafoxidine sulfamate and raloxifene sulfamate are 10 (Ki = 4 micromolar) and 60 times (Ki = 60 nanomolar) respectively more potent than (E)-4-hydroxytamoxifen sulfamate in estrone sulfatase inhibition. The dual inhibitors also inhibit the proliferation of estrogen-dependent breast cancer cell growth stimulated by estrone sulfate.

In conclusion, the newly synthesized inhibitors represent potential agents for the treatment of estrogen-dependent breast cancer.

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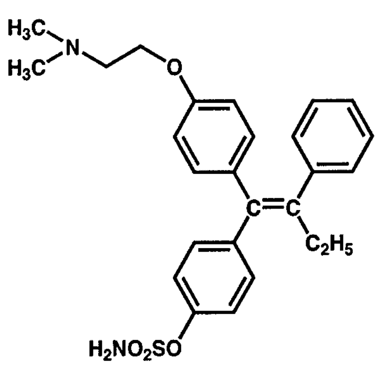
SYNTHESIS AND SULFATASE INHIBITORY ACTIVITIES OF CONFORMATIONAL RESTRICTED ANALOGS OF (E) -4-HYDROXYTAMOXIFEN SULFAMATE

Guo-Hua Chu,JianDong Shi, Zhigen Hu, Heidi Kabler, Kyle W. Selcer and Pui-Kai Li

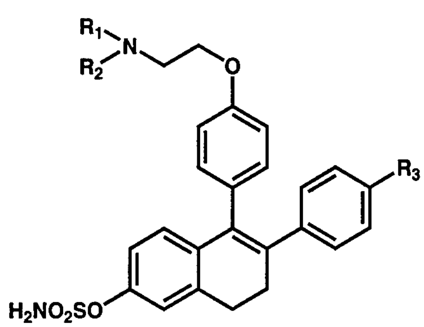
+*Department of Medicinal Chemistry and Pharmaceutics , Mylan School of Pharmacy, Duquesne University, Pittsburgh PA 15282 and Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Columbus OH 43210*

Abstract: Eight conformational restricted analogs of (E)-4-hydroxytamoxifen sulfamate are synthesized as estrone sulfatase inhibitors. All the inhibitors significantly inhibited estrone sulfatase activity. Varying the nature of the substituents in R_3 (H, CH_3, OCH_3, OH) has little effect on the sulfatase inhibitory activity. However, inhibitors with pyrrolidinyl group consistently exhibit higher sulfatase inhibitory activities than the inhibitors with dimethylamino groups.

There is substantial evidence that breast tumors in post-menopausal women accumulate high concentration of estrogens[1,2] and possibly through conversion of estrone sulfate to estrone by the enzyme estrone sulfatase.[3,4] Several estrone sulfatase inhibitors (both steroidal and non-steroidal) have been developed as potential agents for the treatment of estrogen-dependent breast cancers.[5-26] Since the pharmacophore for sulfatase inactivation is a phenylsulfamoyl group, it occurs to us that a potent antiestrogen such as (Z) 4-hydroxytamoxifen can be easily converted to the respective sulfamate analog and becomes potential dual inhibitor (inhibitor with sulfatase inhibitor activity and antiestrogenic activity). Recently, we have synthesized (E) 4-hydroxytamoxifen sulfamate (Fig. 1), the sulfamoylated analog of the potent antiestrogen (Z) 4-hydroxytamoxifen, and demonstrated that it is four fold better than the substrate estrone sulfate in binding to estrone sulfatase.[27] However, the potent antiestrogen (Z)-4-hydroxytamoxifen has been shown in vitro to isomerize into a mixture of Z and E isomer.[28] (E)-4-Hydroxytamoxifen is estrogenic [101, 102].[29,30] Since the conjugation of the hydroxy group in (Z)-4-hydroxytamoxifen with the central double bond is responsible for the facile isomerization [103].[30], one method to fix the configuration of the double bond is incorporating it into a ring such as in nafoxidine. Thus, inhibitors **1 - 8** were synthesized as conformational restricted analogs of (E) 4-hydroxytamoxifen sulfamate.

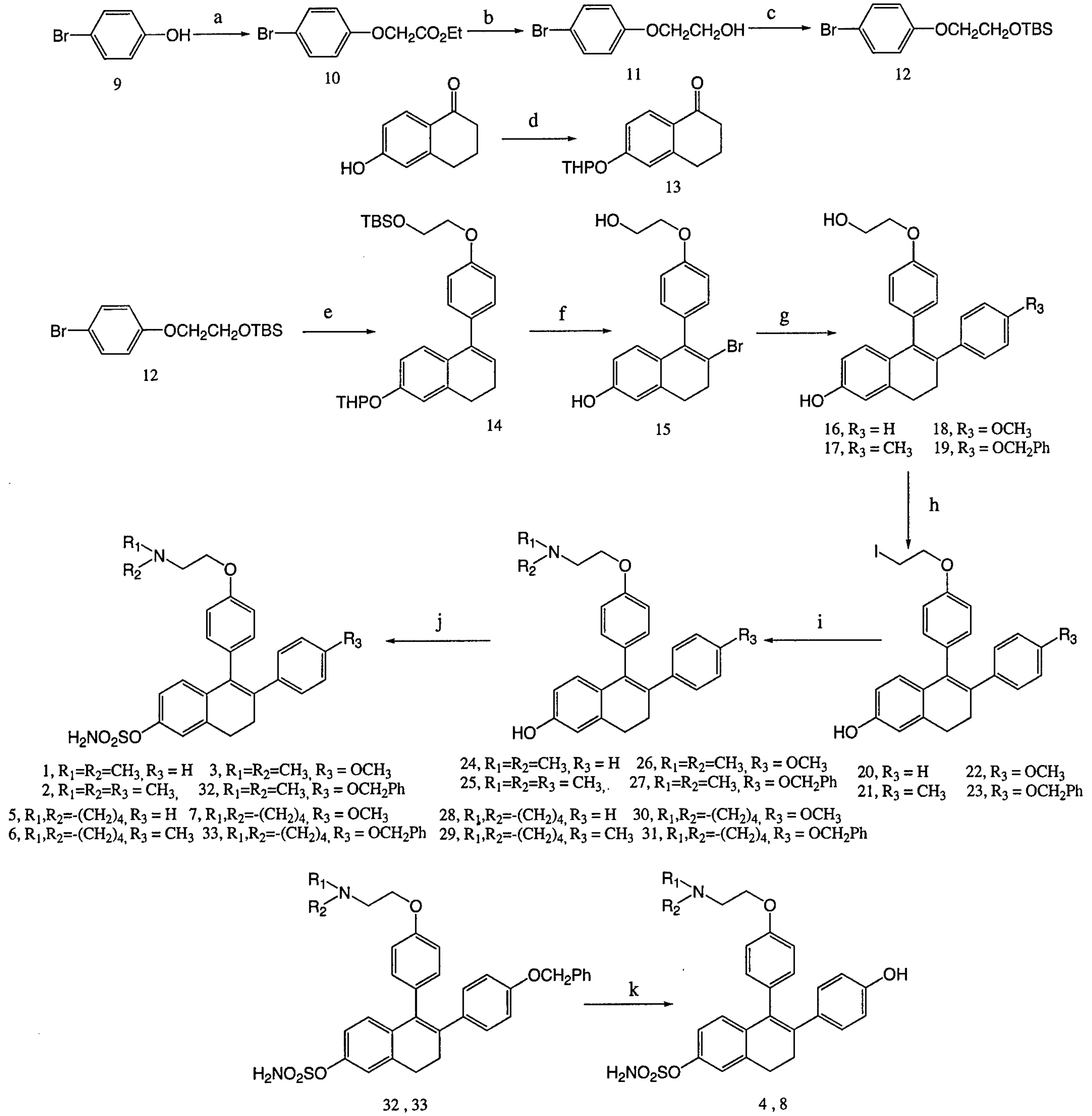


(E)-4-Hydroxytamoxifen sulfamate



Compound	R_1	R_2	R_3
1	CH_3	CH_3	H
2	CH_3	CH_3	CH_3
3	CH_3	CH_3	OCH_3
4	CH_3	CH_3	OH
5	- $(CH_2)_4$ -		H
6	- $(CH_2)_4$ -		CH_3
7	- $(CH_2)_4$ -		OCH_3
8	- $(CH_2)_4$ -		OH

Scheme 1

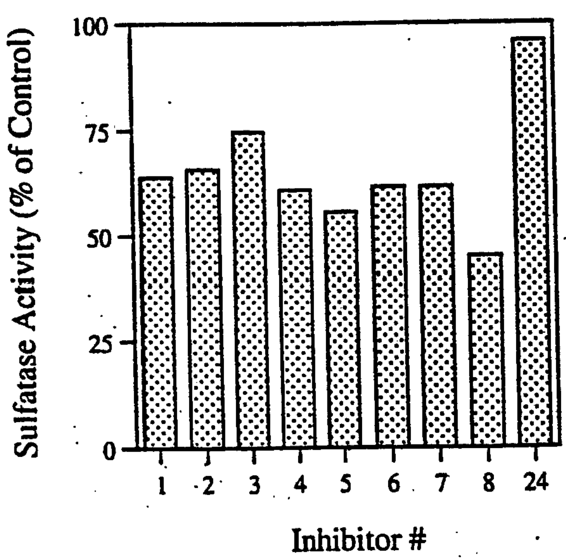


Reagents and Conditions: a. $BrCH_2CO_2Et$, K_2CO_3, acetone, reflux 2.5 h, 99.3 %; b. $LiAlH_4$, THF, r.t, 2 h; c. TBSCl, Imidazole, DMF, r.t, overnight, 96.4 % for 2 steps; d. Dihydropyran, PPTs, CH_2Cl_2, r.t, 2.5 h, 98 %; e. i) n-BuLi, THF, -78°C, 45 min; ii) **13**, -78°C to r.t, 3 h; iii) SiO_2, CH_2Cl_2, r.t, overnight, 65.7% based on **13**; f. i) $C_5H_5N.HBr_3$, CH_2Cl_2, 0°C, 1.5 h; ii) 2N HCl, THF, r.t, 1.5 h, 90.3 %; g. R-Ph-ZnCl (R = H, CH_3, OCH_3, OCH_2Ph), $Pd(PPh_3)_4$, THF, reflux, 2.5 h, 91 - 94 %; h. I_2, PPh_3, Imidazole, CH_2Cl_2, r.t, 40 min, 93 - 95 %; i. $(CH_3)_2NH$ or pyrrolidine, K_2CO_3, THF, r.t, 20 h, 88.3 - 94.1%; j. $ClSO_2NH_2$, 2,6-di-tert-butyl-4-methylpyridine, r.t, 1 h, 91 - 94 %; k. H_2, 10% Pd/C, CH_2Cl_2-CH_3OH (3:1), r.t, 1 h, 79.2 % for **4**, 82 % for **8**.

The synthesis of compounds **1** - **8** is summarized in scheme 1. Reaction of 4-bromophenol **9** with ethyl bromoacetate gave ester **10** (99.3 %). Reduction of **10** with $LiAlH_4$ followed by protection of the resulting alcohol **11** as TBS ether yielded compound **12** (96.4 % for 2 steps). Treatment of **12** with n-butyl lithium , then with ketone **13** which was prepared by tetrahydropyranylayion of 6-hydroxy-1-tetralone (98%), followed by dehydration of the resulting tertiary alcohol with silica gel, afforded olefin **14** (65.7% based on **13**). Bromination of compound **14** with pyridinium tribromide followed by acidic hydrolysis furnished the vinyl bromide **15** (90.3%). Palladium catalyzed coupling[31] of compound **15** with various para-substituted phenyl zinc chlorides which were prepared by the treatment of the corresponding substituted phenylbromides with n-butyl lithium followed by zinc chloride, gave compounds **16** - **19** (91-94%). Iodination of alcohols **16** - **19** with I_2/PPh_3/Imidazole yielded the iodides **20** - **23** (93-95%). Reaction of compounds **20** - **23** with dimethylamine or pyrrolidine gave the corresponding amines **24** - **27** and **28** - **31** respectively (88.3 -94.1%). Sulfamoylation[32] of **24** -**26** and **28** - **30** with sulfamoyl chloride in the presence of hinder base: 2,6-di-tert-butyl-4-methyl pyridine, yielded the target compounds **1** - **3** and **5** - **7** respectively. Compound **4** was synthesized by sulfamoylation of compound **27** to form compound **32** followed by debenzylation through hydrogenation. The synthesis of inhibitor **8** was similar to **4** except compound **31** was sulfamoylated instead.

Inhibitors **1** - **8** can be divided into 2 series. Both series have the same modifications at the para position (R_3) of the 2-phenyl group. Series 1 (inhibitors **1** - **4**) contain the dimethylamino ethyl moiety while series 2 (inhibitor **5** - **8**) have the pyrrolidinyl ethyl moiety. Inhibitors **1** - **8** were tested for their ability to inhibit estrone sulfatase activity of rat liver microsomes at 20 μM concentrations and in the presence of 20 μM substrate estrone sulfate. Table 1 shows the relative inhibition of estrone sulfatase by the inhibitors. All the inhibitors significantly inhibited estrone sulfatase activity. The sulfamate moiety is essential for sulfatase inhibition since compound **24**, the precursor of inhibitor **1**, did not show sulfatase inhibitory activity (Table 1). Varying the nature of the substituents in R_3 (H, CH_3, OCH_3, OH) has little effect on the sulfatase inhibitory activity. However, inhibitors with pyrrolidinyl group (inhibitors **5** - **8**) consistently exhibit higher sulfatase inhibitory activities than the inhibitors with dimethylamino groups (inhibitor **1** - **4**).

Table 1



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